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jc759 U.S. PTO 01/15/00

jc491 U.S. PTO 09/504230 02/15/00

DOCKET NO. : PHOE-0057 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Mike A. Clark

Serial No.: not assigned **Group Art Unit:** not assigned

Filing Date: February 15, 2000 **Examiner:** not assigned

For: MODIFIED TUMOR NECROSIS FACTOR

EXPRESS MAIL LABEL NO: EL531277089US
DATE OF DEPOSIT: February 15, 2000

Box ☒ Patent Application

☐ Provisional ☐ Design

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☒ continuation-in-part of prior application number 09/006,810.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☐ Provisional Application Cover Sheet.

☒ New or Revised Specification, including pages 1 to 22 containing:

☒ Specification

☒ Claims

☒ Abstract

☐ Substitute Specification, including Claims and Abstract.

☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application: ☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____filed _____
_____

_____

_____.

☐ Signed Statement attached deleting inventor(s) named in the prior application.

☐ A Preliminary Amendment.

☒ Three (3) Sheets of ☒ Formal ☐ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____

_____.

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☒ The prior application is assigned of record to Phoenix Pharmacologics, Inc.

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____.

☒ An ☒ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

☒ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☐ Diskette Containing DNA/Amino Acid Sequence Information.

☐ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _____, is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☒ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

_____

_____.

FEE CALCULATION:

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

				SMALL ENTITY		NOT SMALL ENTITY	
				RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION				$75.00	$	$150.00	$
DESIGN APPLICATION				$155.00	$	$310.00	$
UTILITY APPLICATIONS BASE FEE				$345.00	$ 345	$690.00	$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS							
		No. Filed	No. Extra				
	TOTAL CLAIMS	23 - 20 =	3	$9 each	$ 27	$18 each	$
	INDEP. CLAIMS	4 - 3 =	1	$39 each	$ 39	$78 each	$
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$130	$	$260	$
ADDITIONAL FILING FEE					$		$
TOTAL FILING FEE DUE					$411		$

☒ A Check is enclosed in the amount of $ 411.00.

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

☐ The foregoing amount due.

☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.

☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).

☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-

identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: Feb 15, 2000

Dianne Elderkin

Dianne B. Elderkin
Registration No. 28,598

Woodcock Washburn Kurtz
Mackiewicz & Norris LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

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Applicant: Mike A. Clark

Serial No.: not assigned **Attorney's Docket No.: PHOE-0057**

Date Filed: February 15, 2000

For: MODIFIED TUMOR NECROSIS FACTOR

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(d) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am:

( ) the owner of the small business concern identified below:

(XX) an official empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Phoenix Pharmacologics, Inc.
ADDRESS OF CONCERN: 115 John Robert Thomas Drive
Exton, Pennsylvania 19341

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that: (1) the number of employees of the concern, including those of its affiliates, does not exceed 500 persons; and (2) the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **MODIFIED TUMOR NECROSIS FACTOR** by inventor **Mike A. Clark** described in

(XX) specification filed herewith.

( ) application serial no. _____, filed _____.

( ) patent no. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

FULL NAME:

ADDRESS:

( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING John S. Bomalaski
TITLE OF PERSON SIGNING President
ADDRESS OF PERSON SIGNING Phoenix Pharmacologics, Inc.
115 John Robert Thomas Drive
Exton, Pennsylvania 19341

John S. Bomalaski

JOHN S. BOMALASKI

2-14-00

DATE

MODIFIED TUMOR NECROSIS FACTOR

Related Applications

This application is a continuation-in-part of U.S.S.N. 09/006,810, filed January 14, 1998, which claims the benefit of U.S. Provisional Patent Application Serial No. 60/035,521, filed on January 15, 1997.

Field of the Invention

This invention is directed, *inter alia*, to tumor necrosis factor, and several mutant forms of tumor necrosis factor, formulated with polyethylene glycol having a molecular weight in the range of 10,000 to 40,000 and methods for treating tumors using such modified tumor necrosis factor.

Background of the Invention

Malignant melanoma (stage 3) is a fatal disease killing most patients within one year of diagnosis. The incidence of melanoma is rapidly increasing in the United States and is even higher in other countries, such as Australia. Effective treatments for patients suffering from melanoma are urgently needed.

Kidney cancer currently kills approximately 13,000 individuals in the United States each year. This form of cancer is frequently not detected until it is well advanced. The only form of treatment that significantly affects a patient's prognosis is surgical resection of the affected organ. Unfortunately, because this type of cancer is highly metastatic, complete removal of all the metastasis is difficult, if not impossible.

Colon cancer is one of the most prevalent forms of cancer and currently kills approximately 140,000 individuals in the United States each year. Although there have been a large number of traditional chemotherapeutic drugs developed to treat this disease, long term survival (defined as the percentage of patients surviving five years or more) has not appreciably changed in the last four decades. Furthermore, all of the traditional chemotherapeutic drugs developed are highly toxic, have deleterious and often fatal side effects, and are expensive. A curative, non-toxic treatment for this disease is urgently needed.

A hallmark of melanomas, kidney and colon tumors is that these tumors quickly develop resistance to traditional chemotherapies. Even though patients may initially respond to chemotherapeutic treatment, drug-resistant tumors quickly arise and often kill the patient. An alternative way to treat these tumors would be to identify an "Achilles Heel" in the tumors and to develop therapies that would selectively treat that target. One such potential target has been identified. Specifically, it has been noted that all three of these types of tumors require extensive vascularization of each of the metastacies in order for the cancers to grow. Therefore, one would predict that a therapeutic agent which would inhibit the vascularization of these tumors may provide a unique means of treating these tumors.

Tumor necrosis factor (TNF) is a cytokine originally named for its ability to kill tumors. There are at least two different mechanisms by which TNF is believed to kill tumors. First is by a direct effect on the tumor itself. Second, TNF can selectively disrupt the vascularization of tumors, thus depriving the tumor of nutrients and oxygen and in so doing killing the tumor indirectly. This latter mechanism of killing was described in the first scientific publication describing TNF. Carswell and Old reported that the METH A tumor cells were completely resistant to TNF *in vitro*. *J. Proc. Natl. Acad. Sci USA*, **72**:3666-3670 (1975). However, METH A tumors in mice were extremely sensitive to killing by TNF *in vivo*. It was later shown that TNF selectively disrupted the vascularization of these METH A tumors. Subsequently it was later shown that a factor (EMAP 2) is released by some tumors that renders the tumor vasculature susceptible to TNF killing. Thus, TNF can kill some tumors (such as METH A sarcomas) not by directly

killing the tumor cells, but rather by killing the tumors' vasculature that provides the tumor with blood, oxygen and other nutrients necessary to live and grow.

Early clinical trials attempted to utilize TNF as a direct tumoricidal agent. This coupled with the fact that because TNF has a very short circulating half life (less than 20 minutes) in the circulation, extremely high doses of TNF were used which induced "shock"-like symptoms characterized by a precipitous drop in blood pressure and often death of the patient.

An alternative method of using TNF would be to formulate it so that it remains in the circulation longer thus giving it more time to react with (and thus destroy) the vasculature of the tumors. Several other therapeutic proteins which had very short circulating half lives have been formulated with polyethylene glycol (PEG) so that they circulate longer and remain in the vasculature. These proteins include asparaginase, adenosine deaminase, and super oxide dismutase. See, for example, Harras, J.M., in "Polyethylene Glycol Chemistry: Biotechnical and Biochemical Applications," Plenum Press (1992).

Relevant to the invention described here, a group of investigators in Japan (Tsutsumi et. al.) have described that TNF could be formulated with certain PEG and that the resulting material had substantially increased circulating half-life and greater anti-tumor activity. See, Tsutsumi, Y., et al., *Jap. J. Cancer Res.*, **85**:9-12 (1994); Tsutsumi, Y., et al., *Jap. J. Cancer Res.*, **85**:1185-1188 (1994); Tsutsumi, Y., et al., *Jap. J. Cancer Res.*, **87**:1078-1085 (1997). However these investigators used only PEG with a molecular weight of 5000 (PEG5000) attached to the primary amines on TNF with a succinimidyl succinate linker and failed to determine not only the optimal method of attaching PEG to TNF but also the optimal attachment sites on the molecule

Summary of the Invention

It has now been found that TNF modified with polyethylene glycol (PEG) having an approximate weight average molecular weight much higher than that experimented with by Tsutsumi et al., namely, in the range of about 10,000 to about 40,000 and preferably in the range of about 20,000 to about 30,000, is a greatly and surprisingly enhanced tumoricidal agent.

For one thing, the PEG-modified TNF of this invention has a significantly longer circulating half life than the PEG5000-modified TNF of Tsutsumi et al. For example utilizing the technology discovered by Tsutsumi et al., the circulating half life of PEG-TNF was about 2 days. In contrast using the most preferred PEG-modified TNF described herein, the circulating half-life is greater than 16 days (an 8 fold increase).

The PEG-modified TNF of this invention also exhibits significantly and surprisingly enhanced tumoricidal activity compared to either native TNF or to TNF modifed as by Tsutsumi et al. For example, tests indicate that the anti-tumor ED50 of the most preferred embodiment of the invention (TNF modified by PEG-20000 through primary amine groups), is as little as 10-50 IU (0.01 ug), a 2000 fold improvement over the 1000-3000 IU (2 ug) ED50 for native TNF.

Also surprising is the discovery that the modified TNF of this invention, even though it is far more potent at curing tumors and circulates many times longer than native TNF, is actually much safer (and less toxic) to use than the TNF of Tsutsumi et al. Tests indicate that optimal formulation of PEG-TNF circulated 8 times longer, is 2000 fold more potent and about 500 fold less toxic than the PEG-TNF described by Tsutsumi et. al in their publications. Moreover, it has been shown that this optimal formulation methodology can be utilized with TNF of several species (mouse and human) and with both wild type TNF as well as several mutant TNF proteins. Thus the present invention has enormous versatility in being able to be applied to many different TNF molecules.

This invention, therefore, relates to the modified TNF, wherein TNF has been modified by covalently bonding to the TNF, either directly or through a biocompatible linking agent, and preferably through a primary amine on the protein, PEG molecules, each PEG molecule of has an approximate weight average molecular weight in the range of about 10,000 to about 40,000. Preferably, the TNF is modified with five to twelve of the PEG molecules, more preferably, with about five to nine PEG molecules.

This invention also relates to a method of treating a patient suffering from a tumor by administering to said patient a therapeutically effective amount of said modified TNF.

This invention further relates to a method of enhancing the circulating half life of TNF comprising modifying said TNF by covalently bonding to it, preferably

through primary amines on the protein, between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

This invention further relates to a method of enhancing the tumoricidal activity of TNF comprising modifying said TNF by covalently bonding to it, preferably through primary amines on the protein, between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

This invention further relates to a method of enhancing the safety of TNF by covalently bonding to it, preferably through primary amines on the protein, five to twelve PEG molecules each molecule having a molecular weigh of 10,000 to 40,000.

Description of the Drawings

Figure 1 is a graph depicting the circulating half life in mouse serum of native TNF (open circles), SS 5,000 MW PEG-TNF (closed circles), and 20,000 MW PEG-TNF (open triangles).

Figure 2 is a graph depicting the circulating half life in mouse serum of native TNF (open circles), SS 5,000 MW PEG-TNF (closed circles), SS 12,000 MW PEG-TNF (closed triangles), SS-20,000 MW PEG-TNF (open triangles), NHS 12,000 MW PEG-TNF (closed squares), and NHS 20,000 MW PEG-TNF (open squares).

Figure 3 is the sequence of secreted (mature) mouse and human TNF. Position 1 is the N-terminus of the secreted TNF.

Detailed Description of the Invention

"Tumor necrosis factor" or "TNF" as used herein encompasses either naturally derived protein, such as isolated human or mouse TNF proteins, or protein produced using recombinant technology, such as recombinant murine TNF and recombinant human TNF or various TNF mutant proteins. Although the TNF-α protein is preferred, the term "TNF" also encompasses TNF-β protein. The terms also encompass TNF proteins that have been mutated by deletion or alteration of amino acids without significantly impairing biological activity. As non-limiting examples, such mutations

include (reference being made to the sequence of the secreted protein, as illustrated in Figure 3) : the protein in which amino acids 1-9 (MSTESMIRD) of the human secreted protein are deleted; the protein in which lysine at position 166 is changed to alanine; the protein in which lysine at positions 188 and 204 is changed to alanine; and combinations of these mutations.

"Polyethylene glycol" or "PEG" refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chains, represented by the general formula $H(OCH_2CH_2)_nOH$. "Polyethylene glycol" or "PEG" is used in combination with a numeric suffix to indicate the approximate weight average molecular weight thereof of each molecule. For example, PEG 5,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 5,000; PEG 12,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 12,000; and PEG 20,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 20,000. Such polyethylene glycols are available from several commercial sources, and are routinely referred to, as indicated above, by their weight average molecular weights.

"Melanoma" may be a malignant or benign tumor arising from the melanocytic system of the skin and other organs, including the oral cavity, esophagus, anal canal, vagina, leptomeningers, and/or the conjunctivae or eye. The term "melanoma" includes, for example, acral-lentginous melanoma, amelanotic melanoma, benign juvenile melanome, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungual melanoma and superficial spreading melanoma.

"Patient" refers to an animal, preferably a mammal, more preferably a human.

"Biocompatible" refers to materials or compounds which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic and disease states.

"Circulating half life" refers to the period of time, after injection of the modified TNF into a patient, until a quantity of the TNF has been cleared to levels one half of the original peak serum level. Circulating half life may be determined in any relevant species, including humans or mice.

"Covalently bound" as used herein refers to a covalent bond linking the TNF protein to the PEG molecule, either directly or through a linker.

According to this invention, TNF is modified with polyethylene glycol having an approximate weight average molecular weight in the range of 10,000 to 40,000, preferably in the range of 20,000 to 30,000. Generally, polyethylene glycol with a molecular weight of 30,000 or more is difficult to dissolve, and yields of the formulated product are greatly reduced. The polyethylene glycol may be branched or straight chain, but is preferably a straight chain.

The polyethylene glycols may be bonded to the TNF through biocompatible linking groups. As discussed above, "biocompatible" indicates that the compound or group is non-toxic and may be utilized *in vitro* or *in vivo* without causing injury, sickness, disease or death. PEG may be bonded to the linking group, for example, via an ether bond, an ester bond, a thiol bond, or an amide bond. Suitable biocompatible linking groups include, for example, an ester group, an amide group, an imide group, a carbamate group, a carboxyl group, a hydroxyl group, a carbohydrate, a maleimide group (including, for example, succinimidyl succinate (SS), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), succinimidyl succinamide (SSA), or N-hydroxysuccinimidyl (NHS), an epoxide group, an oxycarbonylimidazole group (including, for example, nitrophenyl carbonate (NPC) or trichlorophenyl carbonate (TPC)), a trysylate group, an aldehyde group, an isocyante group, a vinylsulfone group, a tyrosine group, a cysteine group, a histidine group or a primary amine. Preferably, the biocompatible linking group is an ester group and/or a maleimide group and bonds to the TNF through a primary amine on the TNF protein. More preferably, the linking group is SS, SPA, SCM, SSA or NHS; with SS being the most preferred.

Alternatively, TNF may be coupled directly to PEG (i.e., without a linking group) through an amino group, a sulfhydryl group, a hydroxyl group, or a carboxyl group.

Methods for covalently bonding TNF to PEG, directly or via a biocompatible linking group, are known in the art, as described, for example, in Harras, J.M., in "Polyethylene Glycol Chemistry: Biotechnical and Biochemical Applications," Plenum Press (1992), the disclosure of which is herein incorporated by reference. It is

preferred that the TNF protein be covalently bonded to five to twelve PEG molecules. Methods for determining the number of PEG molecules bonded to the protein are known in the art, for example, Habeeb, A.F.S.A., *Anal. Biochem.*, **14**:328-339 (1966); Harras, J.M., *supra.,* herein incorporated by reference. The number of PEG molecules bonded to TNF will vary according to the linking group utilized, the length of reaction, and the molar ratios of TNF and PEG utilized in the reaction.

As one skilled in the art would recognize, the modified TNF of this invention may be administered in a number of ways, for example, orally, intranasally, intraperitoneally, parenterally, intravenously, intralymphatically, intratumorly, intramuscularly, interstitially, intrarterially, subcutaneously, intraocularly, intrasynoially, transepithelially, and transdermally. A therapeutically effective amount of one of the modified compounds of the present invention is an amount effective to inhibit tumor growth, and that amount may vary according to the method of administration. Generally, effective doses should be in the range of about 0.001 to 0.1 mg/kg, once a week. The modified TNF may be formulated with pharmaceutically acceptable carriers and diluents, as known in the art. For example, for intravenous? administration, the modified TNF may be mixed with a phosphate buffered saline solution, or any other appropriate solution known to those skilled in the art, prior to injection. Tests have shown that the modified TNF is particularly effective in treating melanoma, colon cancer, kidney cancer and breast cancer tumors.

The invention is further demonstrated in the following examples, which are for purposes of illustration, and are not intended to limit the scope of the present invention.

TNF used in the experiments described below was of mouse TNF and human TNF or human TNF mutants. The human TNF was produced in *E. coli and Pichea pasatoris,* and murine TNF as well as the human TNF mutants were produced in *Pichea pastoris*. Recombinant TNF was produced in *E. coli* or *Pichea* using methods similar to those described in Pennica, D., et al., *Nature*, **312**:724-729 (1981); Streekishna, K., et al., *Biochemistry*, **28**:4117-4125 (1989). The mouse TNF was produced in *E. coli* and in *Pichea.*

Example 1

Attachment of PEG to TNF

Coupling PEG to TNF was performed using the general methods described in Harras, J.M., cited above. To TNF (1mg/ml in 100 mm phosphate buffer, pH 7.2-7.5), the SS-PEG , SP-PEG or NHS-PEG was added at a 10 to 50 molar excess and mixed for one hour at room temperature. This results in approximately 8-12 PEG molecules being attached to the primary amines of each molecule of TNF. Other PEG linkers and attachment sites required different pH, reaction times and amounts of PEG all of which must be empirically determined. All PEG-TNF formulations were purified by removing unreacted PEG and from the PEG-TNF by ultra filtration using a 100 kDa cut off filter. In each of the modifications referenced in this example, the TNF was modified with five to 15 molecules of PEG.

Purity of the PEG-TNF was assessed by SDS-PAGE and the percent of primary amines modified by this procedure was determined using florescamine as described by S.J. Stocks (Anal. Biochem. **154**:232 (1986)). SDS-PAGE results indicated that very little, if any, native TNF remained in the preparation after pegylation.

Example 2

Specific Activity of TNF

Prior to pegylation (native TNF), both human and mouse was tested for its biological activity using the L929 cytotoxicity assay originally described in the first publication on TNF and in detail below. The specific activity of the TNF was 10^6 I.U. units per milligram. The protein concentration was determined by the method of Bradford. Bradford, M.M., *Anal. Biochem.*, **72**:248-254 (1976). BSA was used as a standard. Purity of the preparation was assessed by SDS-PAGE gel. All preparations used were >99% pure (i.e. less than 1% of native TNF remained in the PEG-TNF.

Example 3

L-929 Cytotoxicity testing of PEG-TNF

The PEG-TNF were examined for in vitro cytotoxic activity using the L-929 cytotoxicity assay performed according to the procedure set forth below. The specific activity of the native TNF starting material was 1.5×10^6 units/mg a value. The specific activity of the PEG-TNF was most often less than one half of the specific activity of the native TNF. This experiment was repeated using a wide variety of PEG molecular weights, methods of attachment (linkers) and sites of attachment (see Table 1).

Table 1

Effects of The Attachment Site, Linker Chemistry and PEG molecular Weight on the Biological Activity of TNF measured In Vitro (L-929 Cytotoxicity of PEG-TNF)

Attachment Site	Linker	Molecular Weight of PEG	% Activity Retained
Primary Amines	SS-PEG	5,000 mw	55
	SS-PEG	12,000 mw	53
	SS-PEG	20,000 mw	56
	SS-PEG	30,000 mw	54
	SS-PEG	40,000 mw	55
	SP-PEG	5,000 mw	51
	SP-PEG	20,000 mw	52
	PEG2-NHS	10,000 mw	49
	PEG2-NHS	20,000 mw	52
	PEG2-NHS	40,000 mw	54
Hydroxyl groups	Epoxy PEG	5,000 mw	38
	Epoxy PEG	8,000 mw	38
	Glycedal ether	5,000 mw	0
Carboxyl groups	Nitro Phenyl	5,000 mw	21
	Trichloro Phenyl	5,000 mw	11
	Tresylate	5,000 mw	8
	PEG aldehyde	5,000 mw	0

Sulfhydral groups	Vinyl sulfone	5,000 mw	12
	Isocynate	5,000 mw	19
	Maleimide	5,000 mw	43

Example 4

Determination of serum half life of PEG-TNF

In order to measure the circulating half life (serum half life) of TNF and PEG-TNF, an ELISA assay for human and mouse TNF obtained from Genzyme was used. The kit was used as suggested by the manufacturer. Mice were injected with either TNF or PEG (100 units) i.p., and approximately 25 µl of serum was collected from retro-orbital bleeds at the times indicated in Fig. 1. A total of 5 mice (female, C57 bl6 mice, 20-25g) were in each group.

The native TNF (open circles) was cleared very fast, and the only data point above baseline was 30 minutes post-injection.

The SS 5,000 MW peg-TNF (closed circles) had a half life of about 4 days. The half life of the 20,000 MW PEG-TNF (open triangles) was > 15 days.

This experiment was repeated using the treatment groups listed below, and the results presented in Fig. 2: native TNF (open circles); SS 5,000 MW PEG-TNF-α (closed triangles); SS 20,000 MW PEG-TNF (open triangles); NHS 12,000 MW PEG-TNF-α (closed squares). The serum half life for the different treatment groups was > 15 days for NHS 20,000 MW PEG-TNF and SS 20,000 MW PEG-TNF-α; approximately 4 days for SS 5,000 MW PEG-TNF; approximately 6 days for SS 12,000 MW PEG-TNF; approximately 8 days for NHS 12,000 MW PEG-TNF; and 30 min post-injection for native TNF. In summary, each PEG-TNF exhibited a much longer half life than native TNF; however, the NHS 20,000 MW PEG-TNF and the SS 20,000 MW PEG-TNF had significantly longer half lives (> 15 days) than the TNF modified with lower molecular weight PEG.

Data from these and other experiments performed with additional PEGs, linkers and sites of attachment are shown in Table 2.

Table 2

Effects of The Attachment Site, Linker Chemistry and PEG molecular Weight on Circulating Half Life and In Vitro Cytotoxicity Using L-929 Cells

Native Human TNF Serum Half-Life = 0.02 days (~20 minutes)

Attachment Site	Linker	Molecular Weight of PEG	Serum Half-Life (days)
Primary Amines	SS-PEG	5,000 mw	4
	SS-PEG	12,000 mw	8
	SS-PEG	20,000 mw	16
	SS-PEG	30,000 mw	17
	SS-PEG	40,000 mw	17
	SP-PEG	5,000 mw	5
	SP-PEG	20,000 mw	8
	PEG2-NHS	10,000 mw	7
	PEG2-NHS	20,000 mw	16
	PEG2-NHS	40,000 mw	18
Hydroxyl groups	Epoxy PEG	5,000 mw	5
	Epoxy PEG	8,000 mw	6
	Glycedal ether	5,000 mw	12
Carboxyl groups	Nitro Phenyl	5,000 mw	5
	Trichloro Phenyl	5,000 mw	5
	PEG aldehyde	5,000 mw	21
Sulfhydral groups	Vinyl sulfone	5,000 mw	3
	Isocynate	5,000 mw	3
	Maleimide	5,000 mw	2

These experiments illustrate that by attaching PEG to the primary amines and using PEG of 20,000 to 30,000 mw, the optimum retention of biological activity measured in vitro (L-929 cytoxicity) and the longest circulating half life in vivo are observed. However, a remaining concern is whether increasing the circulating half life of the PEG-TNF would greatly increase the toxicity of the TNF.

Example 5

Lethality of the PEG-TNF

As a screen, two C57 bl6 mice (female, 20-25 g) were injected intraperitoneally (i.p.) with either native TNF or SS-PEG-TNF and survival of the animals was monitored. The doses used were 1, 5, and 10 thousand units of activity.

With native TNF, the following results were obtained:

10,000 I.U. - both mice dead the next morning

5,000 I.U. - one mouse dead next morning; the second mouse in obvious distress (hair ruffled and little movement) and dead after 2 days

1,000 I.U. - one mouse dead the next morning; the second mouse in distress (hair ruffled and little movement) and in such poor condition after 2 days that it was euthanized

With the SS-PEG-TNF, all mice at all doses remained in good health for two weeks following injection. Behavior was normal, as was eating and drinking. There was no change in coat (fur was not ruffled). All of the mice were euthanized 15 days post-injection.

TNF kills mice by causing an abrupt drop in blood pressure. Blood pressure in mice can be measured using a pressure cuff around the tail, much as blood pressure is measured using a pressure cuff around the arm of a human. Because it has been shown that TNF is more lethal to mice having tumors, than normal mice, the animals used in this experiment were implanted with METH A sarcomas grown to approximately 0.5 cm in diameter. In these experiments we injected mice (5 in each group) with various amounts of TNF or PEG-TNF and the blood pressure was measured 2 hours post treatment (the time of minimum blood pressure following treatment. In Table 3 below, the hypotension ED50 is the amount of TNF or modified TNF that caused 50% of the mice to experience hypotension or shock.

High doses of TNF can kill mice within 2 days following treatment. The amount of TNF required to kill half of the mice is calculated at the LD_{50} (Lethal Dose that kills 50% of the mice). Long Term survival is affected by tumor growth and thus an effective Anti-Tumor dose of TNF Is defined as the lowest dose required to enable a treated mouse to live twice as long as a mouse that does not receive treatment. The

amount of TNF or PEG-TNF required to double the life expectancy (by killing the tumor)of 50% of the animals is presented as the Anti-Tumor Activity ED_{50} in Table 3. All amounts of TNF are expressed as the amount of TNF protein and dose not include the weight of the PEG.

An ideal formulation of TNF would result in a PEG-TNF that exhibiting a high LD50 (be less lethal), a high ED_{50} for blood pressure decrease (not cause hypotension or shock), and a low ED_{50} for Anti-Tumor Activity (that is, be very potent at killing the tumor).

In this experiment, succimimidyl succinamide (SS) linker was used to link PEG 20,000 to the primary amines of mouse, human and several biologically active TNF mutants, to see if this formulation technology could be applied to other forms of TNF. The results from these experiments are summarized in Table 3 below

Table 3.

How does Formulation With PEG Effect TNF Toxicity and Anti-Tumor Activity

Species of TNF	Formulation	LD_{50}	Hypo-tension (ED_{50})	Anti-Tumor Activity (ED_{50})
Murine	Native TNF	2 ug	1 ug	20 ug
	PEG TNF	100 ug	2 ug	0.01 ug
Human	Native TNF	7 ug	1 ug	60 ug
	PEG TNF	300 ug	4 ug	0.005 ug
Human -77-87	Native TNF	60 ug	1 ug	2 ug
	PEG TNF	100 ug	5 ug	0.002 ug
Human 188, 204 K-A	Native TNF	300 ug	100 ug	2 ug
	PEG TNF	300 ug	100 ug	20 ug

Example 6

Antitumor Activity of PEG-TNF

The results presented above indicate that modification of TNF with PEG according to this invention not only reduces the lethality of the TNF, but that especially the TNF modified with PEG having a molecular weight of approximately 20,000 exhibited a surprisingly enhanced circulating half life and surprisingly and significantly enhanced anti-tumor activity.

To test the anti tumor activity of the 20,000 mw PEG-TNF with that of the native TNF and the PEG-TNF described by Tsutsumi et al., a test was carried out utilizing the B16 murine melanoma model. C57 bl6 female mice (20-25 g) were injected with one million B16 melanoma cells, s.q. on flank. The tumors were allowed to grow for one week prior to treatment. There were 5 mice in each treatment group, and animals were treated once a week for three weeks. The number of days the animals survived was noted (the experiment was terminated at 180 days and all animals were euthanized; however the animals that survived this amount of time were all tumor free and in good health), and the results are shown below in Table 4.

Table 4

Effect of Native TNF and PEG-TNF on Survival of Mice Implanted with B16 Melanomas

Treatment Group	Survival Time (days)	Average Survival Time
Saline control	18, 18, 20, 21, 24	20.2 days
Native TNF		
10 IU	17, 18, 19, 21, 21	20.2 days
100 IU	16, 18, 19, 19, 23	19.0 days
SS-PEG 5,000 mw TNF		
10 IU	20, 22, 24, 26, 27	23.6 days
100 IU	21, 22, 24, 26, 27	25.0 days
1000 IU	21, 49, 53, 180, 180	96.6 days

SS-PEG 20,000 mw TNF		
10 IU	38, 180, 180, 180, 180	96.6 days
100 IU	180, 180, 180, 180, 180	180 days
1000 IU	180, 180, 180, 180, 180	180 days

Note that all animals surviving 180 days were devoid of tumors and were euthanized.

Similar experiments were performed using a variety of other tumors including kidney, colon leukemia and breast cancer. Mice were injected with 1 x 10^6 tumor cells and, two weeks later, were injected i.p. with the 20,000 mw PEG-TNF once a week, for three weeks. Cure was defined as the percent of animals surviving five times longer than untreated animals. Results are presented in Table 5 and indicate that the modified TNF of this invention is effective in treating melanoma tumors, kidney tumors, colon tumors, and breast tumors.

Table 5

Identification of tumors most sensitive to TNF

Tumor Type	Cell Line	Dose of PEG-TNF	% cured
Kidney	G401	10 IU	80
		30 IU	80
Colon	HT29	10 IU	40
		30 IU	60
		100 IU	80
Breast	MCF7	10 IU	0
		30 IU	0
		100 IU	20
Brain	SW1088	100 IU	0
Leukemia	L1210	100 IU	0
Hepatoma	Hep3B	100 IU	0

The results presented above are surprising for a number of reasons. First, there was no way to predict that modifying TNF with high molecular weight PEG would increase the circulating half-life of the TNF. Indeed, the clearance rate of proteins in general cannot be predicted based on their molecular weight. Second, although it had been previously shown that proteins modified with PEG circulate longer than proteins without PEG, it had not, to the inventor's knowledge, ever been shown or suggested that simply increasing the molecular weight of the PEG would can have a dramatic effect on the circulating half-life of the PEG protein. Third, it had not been previously shown or suggested that using 20,000 - 30,000 molecular weights of PEG would provide the optimal circulating half life. Fourth, it was unexpected that modification of TNF with high molecular weight PEG, although it decreases the in vivo activity of TNF in killing L-929 cells, would actually enhance the tumoricidal activity of the TNF in vivo. This is particularly surprising in view of the added stearic hindrance expected to be created by the high molecular weight modifier such that it would be unable to interact with TNF receptors. Finally, although one would have predicted that the modified TNF, because of its enhanced circulating half life, would have been even more toxic than the native TNF, this, surprisingly, was not the case.

Table 6, below, provides a comparison of the activity, half life, lethality and anti-tumor activity for native TNF, for PEG5000-modified TNF, as disclosed by Tstusumi et al., and for the preferred embodiment of this invention, PEG20,000-modified TNF.

Table 6

Comparison of the Activities and Toxicities of Various PEG and Native TNF

	NativeTNF	Tsutsumi's PEG-TNF	20,000 mw PEG-TNF
In vitro activity (L-929 Cell cytoxicity)	100 %	56%	54-56%
Circulating half life	20 min	3-5 hours	16-18 days
Lethality in Tumor free mice (LD_{50})	20-70 ug	ND	300 ug
Mice with METH A tumors	1-2 ug	10 ug	300 ug

Dose required to cure 50% of mice with tumors			
METH A sarcoma	>2 ug	10 ug	0.01 ug
B16 Melanoma	>2 ug	10 ug	0.01 ug

Methods and Materials

In Vitro Cytotoxicity Assay

Materials

L929 fibroblasts ATCC #CCL1 NCTC clone 929.

Dulbecco's Modified Essential Medium (DMEM) and Fetal Bovine Serum (GIBCO Laboratories, Grand Island, NY #16000-010)

Recombinant Human Tumor Necrosis Factor-α (TNF-α) (prepared in-house)

Microtiter Plate Reader (Molecular Devices Corp., Menlo Park, CA, Emax)

Method B. Propagation of L929 Fibroblasts:

Cells were grown in DMEM supplemented with 10% Fetal Bovine Serum Incubate overnight in a 37°C, 5% CO_2 humidified incubator.. Cells were planted in a 96 well plates (3,000 cells / well in 0.15 ml of DMEM containing Fetal Bovine Serum. After 24 hours of growth, TNF or PEG TNF was added to the wells and the plates were incubated an additional 24 hours the viability of the cells was determined by adding 20 µl of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (25 mg/ml in phosphate buffered saline pH 7.4) to each well of the culture plate and incubating the cultures at 37°C for four hours. After that time, the culture supernatents were discarded and 150 µl of DMSO was added to each well. The absorbance of each well at 570 nm was determined using a micro titer plate reader. Wells that exhibit an A_{540} closest to 50% of the arithmetic mean of the control are considered to represent 50% lysis (1 unit) of the L929 cells.

Determination of Circulating Half Life of PEG-TNF

Materials

ELISA Kits from Genzyme (Cambridge, MA),

Methods

The Elisa kits were used as suggested by the manufacturer. Serum samples were collected from retro orbital plexus using heparinized 50µl capillary tubes. A pretreatment blood sample was collected just prior to i.v. injection with TNF or PEG-TNF formulations. Additional blood samples were collected at 30 minutes, 24 hours as well as 3, 7, 12 and 15 days post-treatment. The samples were centrifuged and the resulting supernatant was stored frozen at -20°C until being assayed.

WHAT IS CLAIMED IS:

1. Modified TNF, comprising TNF covalently bound to between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

2. The modified TNF of Claim 1 wherein said PEG is covalently bound to primary amine groups on said TNF through a biocompatible linker and where said PEG has an approximate weight average molecular weight in the range of about 20,000 to about 30,000.

3. The modified TNF of Claim 1 wherein said linker is selected from the group consisting of succinimidyl succinate, succinimidyl proprionate, and N-hydroxy succinimidyl.

4. The modified TNF of Claim 2 wherein said linker is selected from the group consisting of succinimidyl succinate, succinimidyl proprionate, and N-hydroxy succinimidyl.

5. The modified TNF of Claim 1 wherein said TNF is TNF-α.

6. The modified TNF of Claim 1 wherein said TNF is isolated human TNF.

7. The modified TNF of Claim 1 wherein said TNF is recombinant human TNF.

8. The modified TNF of Claim 1 wherein said TNF is human TNF mutated by deleting amino acids 1-9 of the mature TNF protein.

9. The modified TNF of Claim 1 wherein said TNF is human TNF mutated by changing the lysine at position 166 of the mature protein to alanine.

10. The modified TNF of Claim 1 wherein said TNF is human TNF mutated by changing the lysine at positions 188 and 204 of the mature protein to alanine.

11. Mutated TNF, comprising human TNF in which lysine at one or more of positions 166, 188 and 204 is changed alanine.

12. The mutated TNF of Claim 11 in which lysine at position 166 is changed to alanine.

13. The mutated TNF of Claim 11 in which lysine at both of positions 188 and 204 is changed to alanine.

14. A method of enhancing the circulating half life of TNF while reducing its toxicity comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

15. The method of Claim 14 in which said PEG is covalently bound to primary amine groups on said TNF through a biocompatible linker and where said PEG has an approximate weight average molecular weight in the range of about 20,000 to about 30,000.

16. A method of enhancing the tumoricidal activity of TNF comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules each molecule having an approximate molecular weight of 20,000 to 30,000.

17. The method of Claim 16 in which said PEG is covalently bound to primary amine groups on said TNF through a biocompatible linker and where said PEG has an approximate weight average molecular weight in the range of about 20,000 to about 30,000.

18. A method of inhibiting tumor growth in a patient suffering from a tumor comprising administering to said patient a therapeutically effective amount of the modified TNF of Claim 1.

19. A method of inhibiting tumor growth in a patient suffering from a tumor comprising administering to said patient a therapeutically effective amount of the modified TNF of Claim 2.

20. The method of Claim 19 wherein said tumor is a melanoma.

21. The method of Claim 19 wherein said tumor is a colon cancer.

22. The method of Claim 19 wherein said tumor is a kidney cancer.

23. The method of Claim 19 wherein said tumor is a breast cancer.

ABSTRACT OF THE INVENTION

Modifying TNF with polyethyleneglycol (PEG) having an approximate weight average molecular weight in the range of about 10,000 to about 40,000, preferably in the range of about 20,000 to 30,000, significantly increases the circulating half-life of the TNF while not increasing its toxicity. As a result, lower doses of the TNF may be administered to effectively treat tumors with fewer, accompanying adverse side effects to the patient.

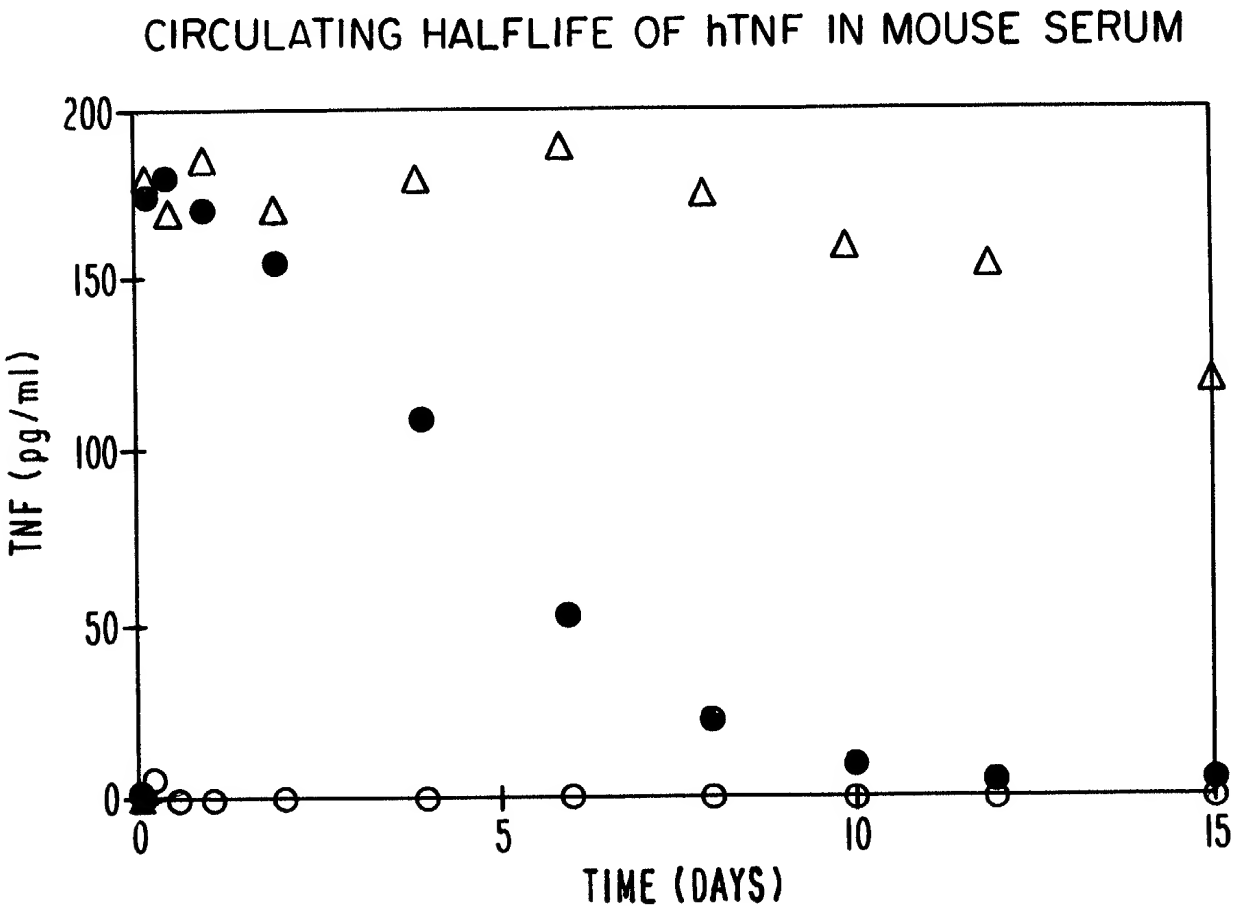
CIRCULATING HALFLIFE OF hTNF IN MOUSE SERUM
200
150
100
50
0
TNF (pg/ml)
0
5
10
15
TIME (DAYS)

Fig. 1

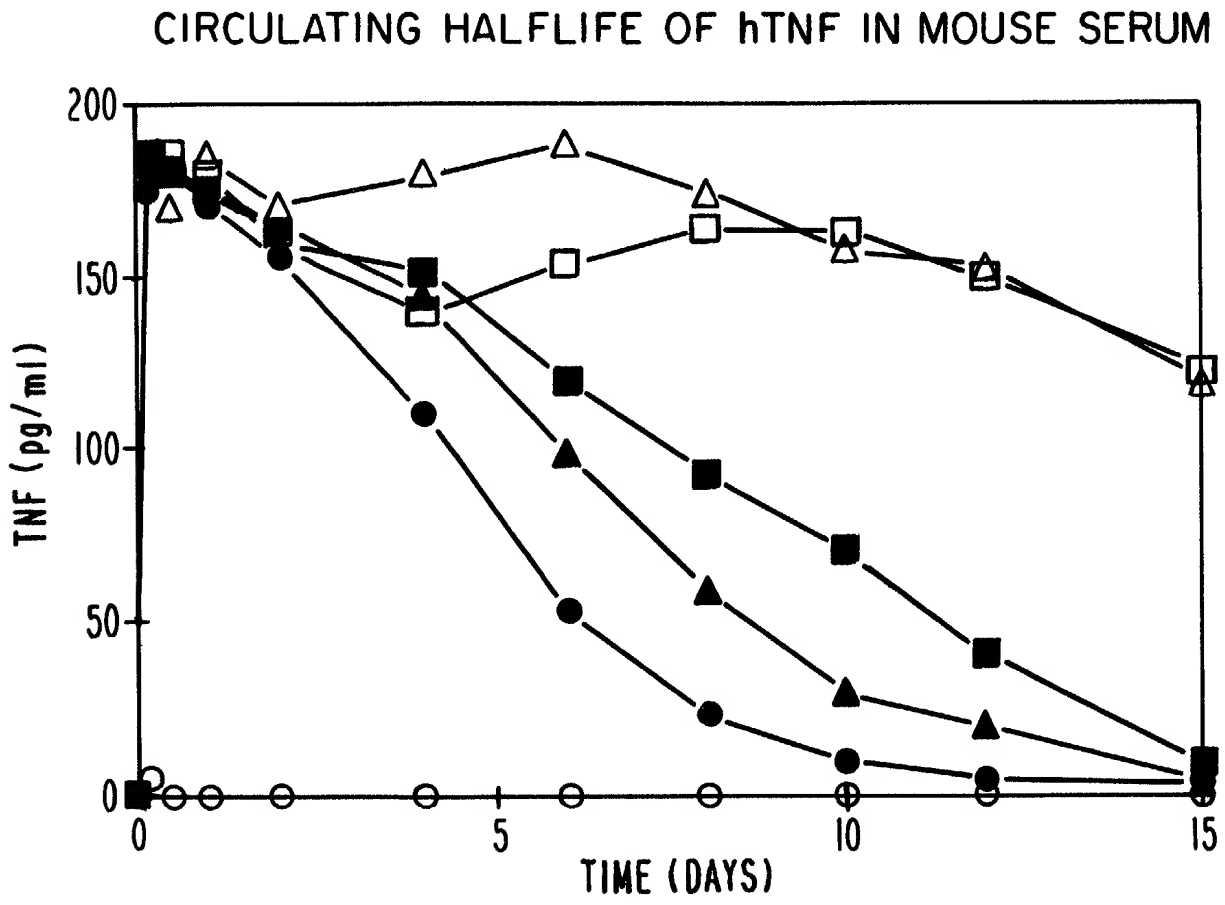
CIRCULATING HALFLIFE OF hTNF IN MOUSE SERUM
200
150
100
50
0
TNF (pg/ml)
0
5
10
15
TIME (DAYS)

Fig. 2

FIGURE 3

Sequence of the Secreated (mature) Mouse and Human TNF Protein

```
Mouse       1 MSTESMIRDVELAEEALPQKMGGFQNSRRCLCLSLFSFLLVAGATTLFCLLNFGVIGPQR
Human       1 MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQR
              **********
                        10

Mouse      61 DEKFPNGLPLISSMAQTL--------------TLTNHQVEEQLEWLSQRANALLANGMDL
Human      61 EE-FPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVEL

Mouse     107 KDNQLVVPADGLYLVYSQVLFKGQGCPD-YVLLTHTVSRFAISYQEKVNLLSAVKSPCPK
Human     120 RDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQR
                                                          A
                                                        166

Mouse     166 DTPEGAELKPWYEPIYLGGVFQLEKGDQLSAEVNLPKYLDFAESGQVYFGVIAL
Human     180 ETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL
                      A               A
                    188             204
```

DOCKET NO. PHOE-0057 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Mike A. Clark **Group Art Unit: not assigned**

For: MODIFIED TUMOR NECROSIS FACTOR **Examiner: not assigned**

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☒ is attached hereto.

☐ was filed on _____ as Serial No. _____ .

☐ said application having been amended on _____ .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign**

application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned
09/006,810	January 14, 1998	Pending

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed
60/035,521	January 15, 1997

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Dianne B. Elderkin Reg. No. 28,598

Address all telephone calls and correspondence to the first-listed attorney of record at:

WOODCOCK WASHBURN KURTZ
MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone No.: **(215) 568-3100**
Facsimile No.: **(215) 568-3439**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name Mike A. Clark	_____ Signature
Mailing Address: 1276 Scoville Road Lexington, KY 40502	Date of Signature: _____
City/State of Actual Residence Lexington, KY 40502	Citizenship: United States

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